Fluorescent Resonance Energy Transfer: from sorter to benchtop
Reconfiguring the Cyan benchtop analyzer to perform CFP-YFP FRET analyses

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Protein interactions at the molecular level can be measured using a pair of fluorescently labeled proteins that have overlapping emission and excitation spectra. When the donor is excited, it fluoresces and gives off energy in within the excitation spectra of the acceptor, and this results in the donor protein being directly excited by the energy release of the acceptor protein. This resultant process of energy transfer from a donor molecule to an acceptor molecule is termed fluorescent resonance energy transfer (FRET).

The phenomenon of fluorescence resonance energy transfer (FRET) was first observed by Jean Perrin in the 1918, and was theoretically explained and named by Theodor Förster in 1948 [1-3]. FRET can only occur when the following two conditions are met: 1.) the emission spectrum of the donor significantly overlays the absorption spectrum of the acceptor and 2.) the distance between the donor and acceptor molecules is less than 100 angstroms (Å). As a result of FRET, the process results in a net energy loss from the donor via excitation - or quenching - and a net energy gain by the acceptor. The actual efficiency of the transfer of this energy is inversely proportional to the sixth power of the distance (γ) between the donor and the acceptor.

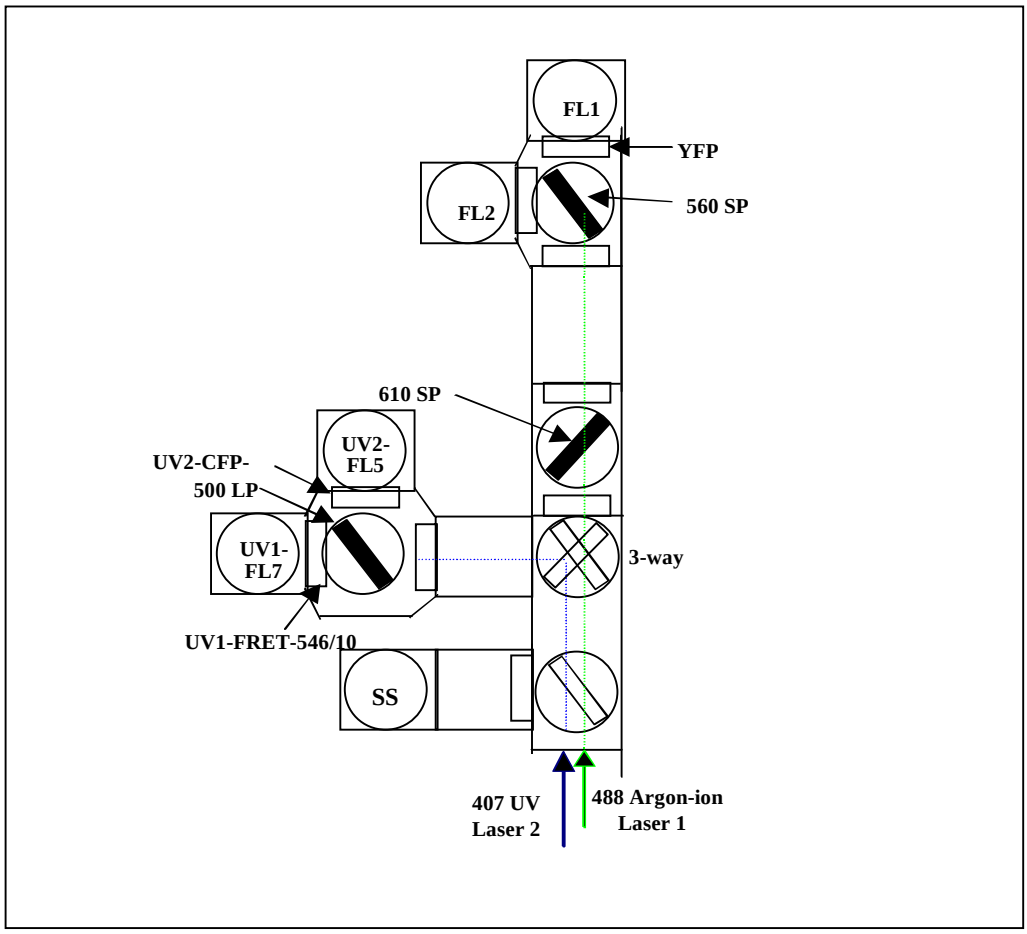
Due to its high sensitivity to measure molecular distance, FRET has been extensively used in biomedical research to determine molecular interactions. With the increased availability of green fluorescent proteins (GFP) and its derived mutants (cyan fluorescent protein, yellow fluorescent protein) as well as the structurally unrelated red fluorescent protein (RFP), the study of *in vivo* protein-protein interactions is becoming increasingly more practical. Although EGFP is very bright, the molecule favorably forms weak dimers[4]; its derived forms - CFP and YFP - are prevented from dimerizing by exchanging any one of three hydrophobic residues believed to be at the dimer interface

[Ala [206], Leu [221] or Phe [223]] with a positively charged residue [5,6]. Because of this, a CFP-YFP FRET pair has classically been the most useful and successful in exploring *in vivo* protein-protein interaction.

This type of analysis has typically been performed on cell sorters, as these instruments have usually been the only ones capable of providing the dual laser lines and combinations of filters necessary to detect FRET. CFP is excited by a 405/7 laser line (or 413 – something around this range of CFP excitation), with YFP being excited by a standard 488 laser. FRET is detected in a spatially distinct fluorescent detector that typically abuts the CFP detection channel. The best combination of filters to detect FRET is a 480/30 bandpass filter for CFP, and a pair of 546/10 bandpass filters for the YFP and FRET channels. Using filters other than the 546/10 filters has required us to use very high percentages of compensation and requires extensive set-up time – therefore, we highly recommend this filter set. A 500LP (or 500 SP) dichroic filter is commonly used in the CFP/FRET PMT hub. The dual laser (407 and 488) system used on a FACSVantage cell sorter is depicted below in figure 1 [7].

FIGURE 1

FACSVANTAGE FILTER CONFIGURATION FOR CFP-YFP FRET DETECTION



Optical bench configuration for performing FRET analysis on the FACSVantage. The 407nM UV laser is used to excite CFP; detection is in FL5 using a 480/30 bandpass filter. The 488nM argon laser is used to excite YFP; detection is in FL1 using a 546/10 bandpass filter. CFP-->YFP FRET is detected in FL7 using a 546/10 bandpass filter. Dichroic filter specifications are noted in the diagram.

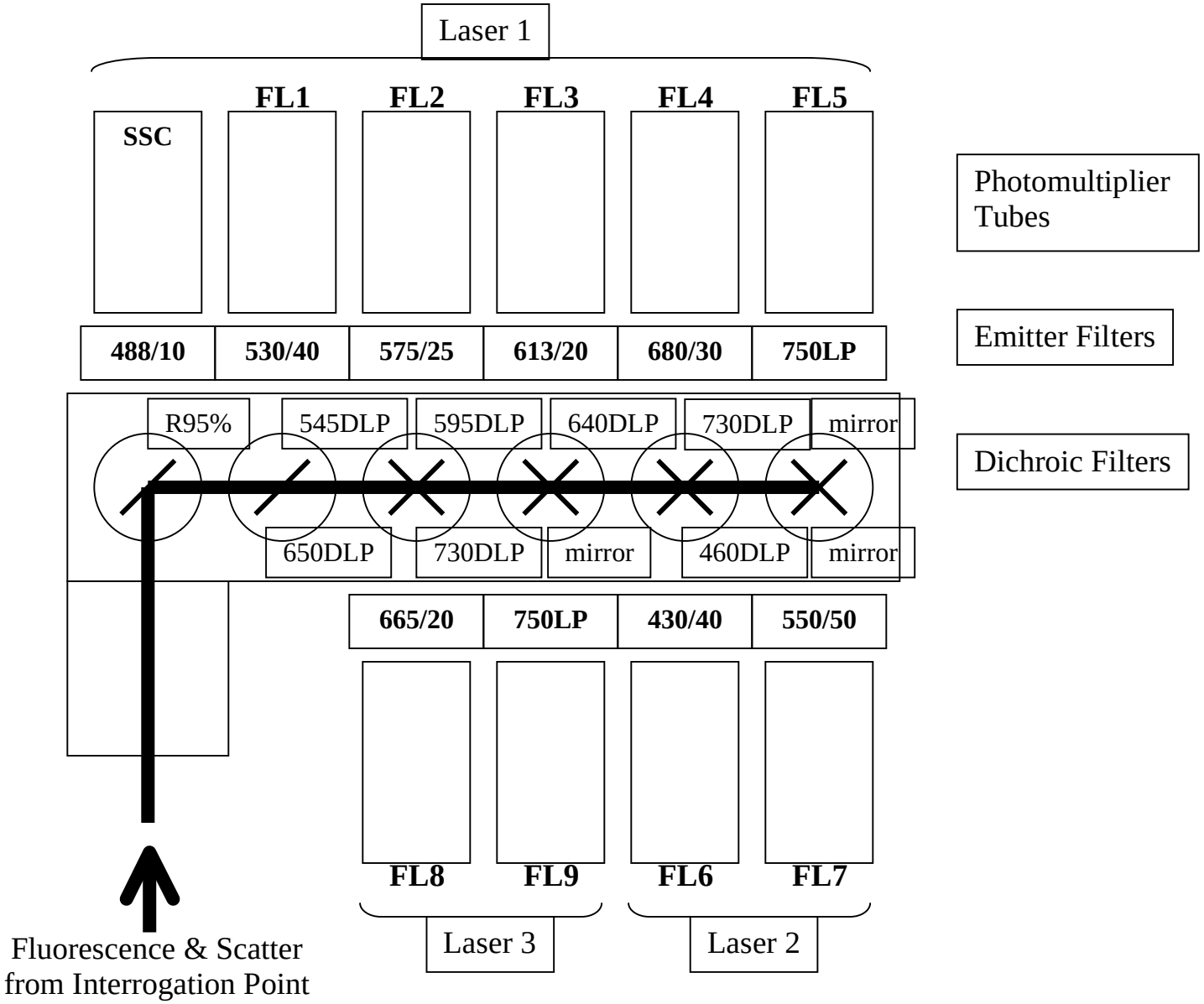
Recent developments in the realm of benchtop analyzers have allowed the technology of FRET to be moved from a cell sorting machine to a steady-state laser cytometer. The advantages of this change is that the sorter can now be dedicated to sorting, rather than FRET analysis, and that the bench top system does not have to be aligned from day to

day. This latter point further minimizes machine variability and standardizes results to a greater degree.

The set up below compares the normal filter configurations for the Cytomation Cyan (Figure 2A) and the appropriately altered filter configuration for performing CFP-YFP FRET analyses on this bench top flow cytometer. CFP-->YFP FRET can be done on the Cyan LX using the standard filter configuration shown in figure 2A, but because of our experience with this filter set on the FACSVantage, we preferred to change the change the filter configurations to facilitate greater ease in compensation when performing this FRET using CFP and YFP. To accomplish this change in bandpass filters and dichroic filters requires an investment of approximately $1,000.00 (in addition to the cost of the machine). The modified Cyan configuration is provided in figure 2B, with filters that necessitate changing being highlighted in color.

FIGURE 2A

STANDARD CYAN LX CONFIGURATION

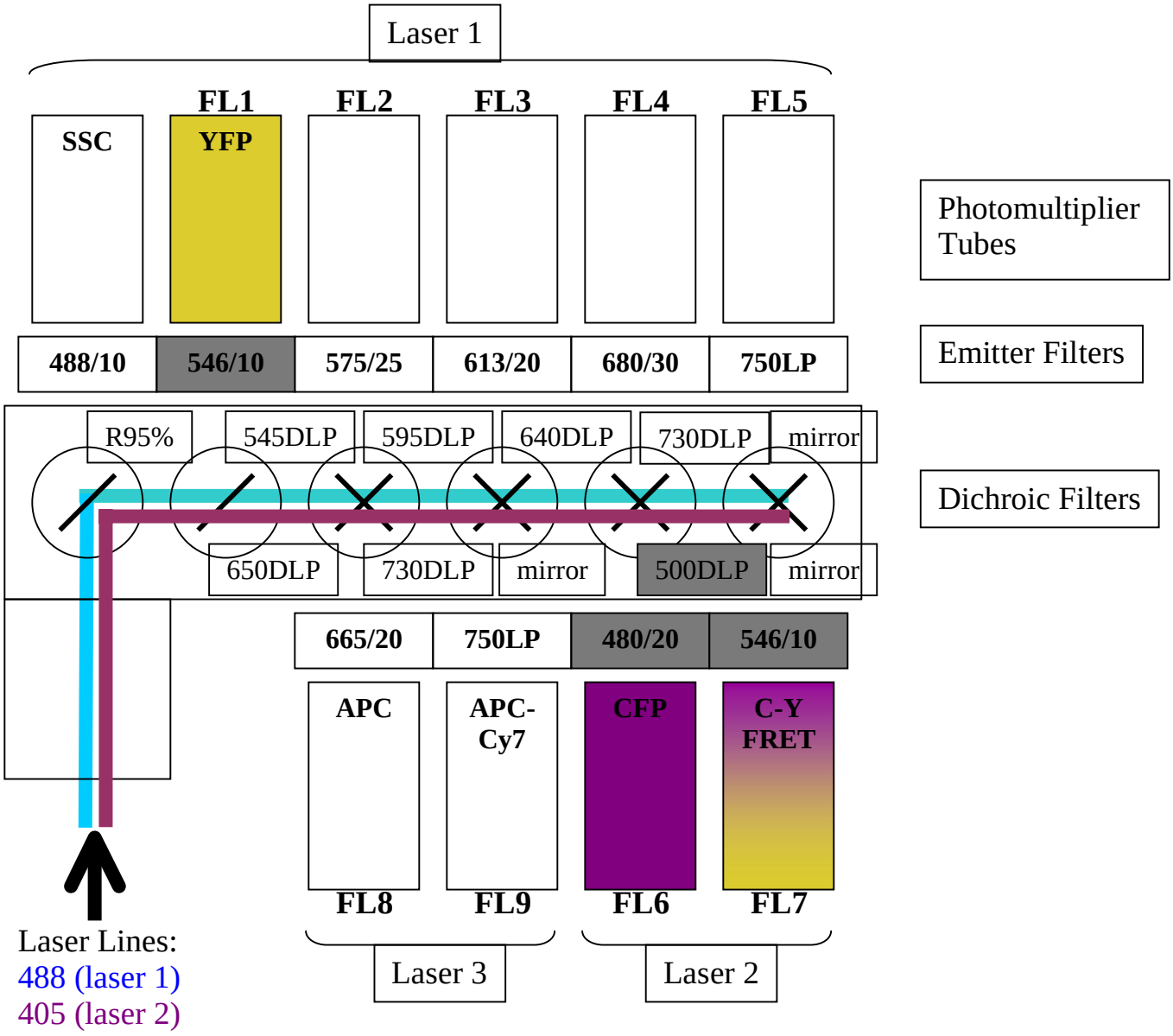


Optical configuration of the Cyan LX. Five photomultiplier tubes are used for 488nM excitation/emission detection; two for th3 633 He-Ne line (laser 2); and two for the 407 violet line (laser 3). Dichroic and bandpass filters are noted in the diagram.

FIGURE 2B

CFP-YFP FRET CYAN CONFIGURATION

(Filters that need to be changed are highlighted in gray; PMTs of interest are colored in cyan, yellow, or a combination of the two colors [5])



Reconfiguration of the standard optical bench on the Cyan LX used to detect CFP-->YFP FRET. PMTs of interest are colored (CFP, cyan; YFP, yellow; FRET, cyan/yellow mix). Dichroic and bandpass filters that need to be changed are highlighted in gray.

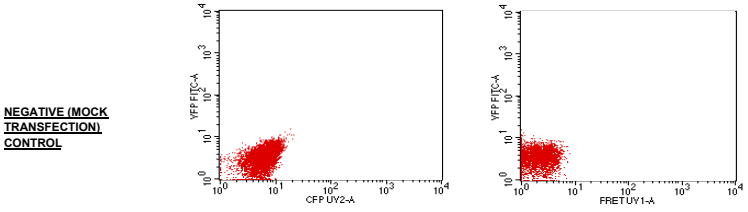
As with any experiment, controls are essential for FRET. If performing a CFP-YFP FRET experiment, one should have at least the following controls:

1. Non-fluorescent protein mock transfection
2. CFP transfection only
3. YFP transfection only
4. CFP-YFP fusion protein transfection
5. CFP-YFP co-transfection with protein that will not interact (a CFP and YFP positive control in the same cells that will not produce a FRET signal – for instance, CFP in the cytoplasm, YFP in the nucleus).

These controls are presented below in Figure 3, to provide a representative idea of the appearance of control samples.

FIGURE 3

REPRESENTATIVE CONTROLS NECESSARY FOR CFP-->YFP FRET ANALYSES



Row 1 [negative/mock transfection control] shows mock transfection control (no CFP, no YFP); row 2 [CFP only] shows cells transfected with a CFP vector only; row 3 [YFP only] is cells transfected with a YFP vector only; row 4 [CFP/YFP non-interacting – no FRET] shows cells tranfected with both CFP and YFP vectors that are compartmentalized in different parts of the cell - these proteins do not interact – hence, there is no FRET; row 5 [CFP/YFP fusion – FRET positive control] shows cells transfected with CFP and YFP vectors that are linked and have a robust interaction that leads to a strongly positive FRET signal.

In summary, FRET can now be performed on a bench top analyzer with the appropriate optics configurations. We have shown the optical configurations for the

classical manner to analyze FRET, on a FACSVantage cell sorter. After providing a detailed configuration for this procedure on the FACSVantage, we described the modifications necessary to perform these analyses on the DakoCytomation Cyan benchtop analyzer. Finally, we provide representative data plots for people unfamiliar with FRET to use as a guide when performing this type of analysis. As the field of flow cytometry expands further, it is likely that new, exciting methodology and machine configurations will be added to further enhance the feasibility and ease - as well as biological applicability - of fluorescent resonance energy transfer.

References

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